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Cdc53p Acts in Concert with Cdc4p and Cdc34p To Control the G_1-to-S-Phase Transition and Identifies a Conserved Family of Proteins

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Regulation of cell cycle progression occurs in part through the targeted degradation of both activating and inhibitory subunits of the cyclin-dependent kinases. During G_1, *CDC4*, encoding a WD-40 repeat protein, and *CDC34*, encoding a ubiquitin-conjugating enzyme, are involved in the destruction of these regulators. Here we describe evidence indicating that *CDC53* also is involved in this process. Mutations in *CDC53* cause a phenotype indistinguishable from those of *cdc4* and *cdc34* mutations, numerous genetic interactions are seen between these genes, and the encoded proteins are found physically associated in vivo. Cdc53p defines a large family of proteins found in yeasts, nematodes, and humans whose molecular functions are uncharacterized. These results suggest a role for this family of proteins in regulating cell cycle proliferation through protein degradation.

Progression through the cell cycle in the yeast *Saccharomyces cerevisiae* requires the activity of a protein kinase whose catalytic subunit, p34, is encoded by the *CDC28* gene (for a review, see reference 36). The Cdc28p protein kinase is a member of a large family of highly related protein kinases known as cyclin-dependent kinases, and the specific role of this protein kinase at each discrete stage of the cell cycle is defined by the cyclin subunit with which it is associated. Accordingly, important regulation of cell cycle progression depends on the accumulation and degradation of different cyclins during the different phases of the cell cycle (for a review, see reference 36). Late in the G_1 phase, Cdc28p is found complexed with the G_1 cyclins Cln1p, Cln2p, and Cln3p (9, 46, 50), and this activity is required for executing Start. Activation of Start is marked by the initiation of several events: spindle pole body (SPB) duplication, bud emergence, and activation of SBF- and MBF-dependent transcription (5, 24, 35). At the same time, or closely thereafter, Cdc28p becomes associated with the Clb5p and Clb6p cyclins. Clb5p and Clb6p, although not required for viability, are necessary for the timely initiation of DNA replication (10, 42).

The activity associated with Cdc28p at the various steps in the cell cycle is also modulated by inhibitory proteins that bind to specific cyclin-kinase complexes (for reviews, see references 43 and 44). The activities of such inhibitory proteins are themselves controlled through degradation (32, 41). For example, prior to S phase, Clb5p-Clb6p kinase complexes are prevented from functioning by being bound to the inhibitory Sic1 protein (41). At the initiation of S phase, these complexes undergo activation by the degradation of Sic1, leading to DNA replication (41). If the degradation of Sic1p is prevented, entry into S phase is blocked and cells remain at the G_1-to-S-phase boundary (41).

CDC34 is also critical for the progression of yeast cells into S phase (12). In the absence of *CDC34* function, cells perform Start-related events but fail to perform subsequent events such as the replication of nuclear DNA, spindle formation, and cytokinesis (5, 6). The *CDC4* gene product has also been shown to be required for these events (5, 14). In fact, cells mutant for *cdc4* or *cdc34* are thus far phenotypically indistinguishable from one another.

Both *CDC4* and *CDC34* have been characterized at the molecular level (12, 52). *CDC4* encodes a protein containing WD-40 repeats, the specific role of which in cell cycle control remains undefined. *CDC34* encodes a ubiquitin-conjugating enzyme that catalyzes the formation of a polyubiquitin chain on several substrate proteins (3, 8, 12). The presence of polyubiquitin as a posttranslational modification serves in targeting proteins for degradation by the ATP-dependent protease called the proteasome (for a review, see reference 16). Cdc34p, as well as Cdc4p, has been shown to be involved in the destruction of multiple cell cycle regulators, including Cln2p, Cln3p, Far1p, and Sic1p (8, 26, 30, 41, 47, 51). Furthermore, in the absence of Cdc34p or Cdc4p activity, the Cln kinase complexes required for Start remain active (47), but the Clb5p-6p kinase complexes that are required for the initiation of S phase are not activated (41).

Although the evidence suggesting that the Cdc34p-mediated destruction of both the cyclins and inhibitory proteins is a key regulatory event of the G1-to-S-phase transition is compelling, the mechanisms by which the activity of Cdc34p is controlled and by which it recognizes its substrates have remained obscure. We describe here the identification of a gene, *CDC53*, that is required at the same stage of the cell cycle as *CDC34*,

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
5566-1-1	*MAT*α *cdc39-1 trp1 lys2 ade2 his4 cry1 SUP4*	L. Hartwell
5579-5-2	*MAT*α *cdc36-16 trp1 lys2 ade2 his4 cry1 SUP4*	L. Hartwell
6353-4-1	*MAT*α *cdc4-1 trp1 lys2 ade2 his4 cry1 tyr1 SUP4*	L. Hartwell
6353-14-3	*MAT***a** *cdc4-1 trp1 lys2 ade2 his4 cry1 SUP4*	L. Hartwell
7414-2-3	*MAT*α *cdc28-1 leu2-3,112 ura3-52*	J. Konopka
H122-12-3	*MAT*α *cdc10-1 ura1 ade1 ade2*	L. Hartwell
H2C2A1	*MAT***a** *cdc2-2 his7 ura1*	L. Hartwell
H3C1B5	*MAT*α *cdc3-1 his7 ura1*	L. Hartwell
JPT175	*MAT***a** *cdc53-1*	This study
JPTA1528	*MAT*α *cdc53-4*	This study
JPTA1529	*MAT*α *cdc53-3*	This study
KJB1	*MAT***a** *ade2 trp1 ura3-52 mip1*::*URA3 cdc53-2*	This study
MG-F	*MAT*α *cdc53-1 trp1*	This study
MGG3	*MAT*α *ura3-52 leu2-3,112 his3 can1* *MAT***a** *ura3-52 leu2-3,112 his3 can1*	T. Petes
MGG10	*MAT***a** *cdc53-1 ura3-52 trp1 ade2*	This study
MGG11	*MAT*α *cdc34-1 ura3-52 his3*	This study
MGG12	*MAT*α *cdc53-1 trp1 his3 ade2 ura3-52*	This study
MGG15	*MAT***a** *cdc34-1 ura3-52 his3*	28
MGG47	MG-F × YPH52	This study
MGG48	YPH52 × YPH54	This study
SJ1012-4	*MAT***a** *cdc4-5 leu2-3,112 ura3-52 lys2 ade1 his7*	21
SJ1026-7B	*MAT***a** *cdc4-3 leu2-3,112 ura1 trp1 lys2 his7*	21
SJ1026-1B	*MAT*α *cdc4-3 leu2-3,112 ura1 trp1 lys2 his7*	21
SJ1078-2B	*MAT***a** *cdc4-3 leu2-3,112 ura3-52 trp1 lys5 ade2 ade6*	21
SJ1080-4D	*MAT***a** *cdc53-1 leu2-3,112 ura3-52 ade2 his7*	21
SJ1080-6C	*MAT*α *cdc53-1 leu2-3,112 ura3-52 ade2*	21
SJ1080-8C	*MAT*α *cdc53-1 leu2-3,112 ura3-52 his7*	21
SJ1098-3D	*MAT***a** *cdc34-2 leu2-3,112 ura3-52 trp1*	21
ts328	*MAT***a** *ade1 ade2 ura1 tyr1 lys2 his7 gal1 mnn1 cdc53-2*	This study
WX70.3c	*MAT***a** *ade2 cdc53-2*	This study
Y382	*MAT*α *ade2 ade3 ura3 leu2 trp1*	A. Bender
YL10-1	*MAT***a** *cdc34-2 ura3-52 leu2*Δ*-63 his3*Δ	28
YPH52	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1 his3-200*	P. Hieter
YPH54	*MAT*α *ura3-52 lys2-801 ade2-101 trp1 his3-200*	P. Hieter

and we present evidence that Cdc34p acts in concert with Cdc4p and Cdc53p to execute its cell cycle function. Furthermore, the identification of genes encoding a large family of Cdc53p-related proteins in higher organisms suggests that the control of cell cycle progression in higher cells utilizes similar mechanisms and that these Cdc53p-like proteins also mediate ubiquitin-dependent protein degradation.

MATERIALS AND METHODS

Yeast strains and media. Yeast strains used in this study are described in Table 1. Strains were grown by using standard media and conditions (13, 37) at the indicated temperatures. Yeast transformations and genetic manipulations used standard protocols (37). Yeast mutant collections and genetic screening strategies have been described elsewhere (1, 20, 49).

Flow cytometry and microscopy techniques. Yeast cells were grown under standard conditions at 23°C overnight to a density of 5×10^6/ml and then shifted to 36°C for 3 h. Cells were then prepared for flow cytometry or electron microscopy as described previously (49). For differential interference contrast and fluorescence microscopy, cells were fixed, stained with propidium iodide as described for flow cytometry, and visualized in a Nikon FXA microscope as described previously (11). Images were recorded by using a Pulnix TM-745 camera and an Apple Macintosh Quadra 700 personal computer. Images were analyzed by using the NIH Image 1.53b2 software.

Plasmids. Plasmid manipulations used standard protocols (38). E3a was recovered as a plasmid that could complement the temperature sensitivity of *cdc53-1*. Plasmid E3a contains an 8.2-kb DNA fragment isolated from a plasmid library of *Sau*3AI-partial-digestion fragments cloned into YEp24 (7). Plasmids pGEM53-8 and pGEM53-9 contain a 3.6-kb *Eco*RI DNA fragment encompassing the *CDC53* gene ligated into the *Eco*RI site of pGEM-3 (Promega Corp.) in the two possible orientations. Plasmid pCDC53-9 contains a 7.0-kb *Sph*I DNA fragment from E3a cloned into the *Sph*I site of YRp7, and plasmid pCDC53-11 contains a 3.3-kb *Apa*I-*Sph*I DNA fragment from E3a cloned into the *Sal*I-*Sph*I sites of YRp7 (45). Plasmid pGEM53-ΔBglII::*HIS3* was constructed by digesting plasmid pGEM53-8 with *Bgl*II and ligating it to a *HIS3*-containing 1.7-kb *Bam*HI DNA fragment from CMp170 as described previously (12). This construction replaces 1.6-kb of the *CDC53* coding sequence with the *HIS3* gene.

Plasmids pFUS34, pYL150, pSJ101, and pSJ4101 have been described previously (11, 21, 28). In pFUS34 and pSJ4101, *CDC34* and *CDC4*, respectively, can be expressed from their native promoters or from the *GAL10* promoter. In pYL150, the native promoter of *CDC34* is missing. The 2.6-kb *Apa*LI-*Cla*I DNA fragment from pGEM53-8 was ligated into the *Sal*I-*Xho*I sites of pSJ101 or the *Eco*RI site of pYcDE-2 (kindly provided by B. D. Hall, University of Washington) (after filling in) to create pFUS53-3 and pYcDE-53, respectively. Plasmid pFUS53-3 allows *CDC53* to be expressed from its own promoter or the *GAL10* promoter, whereas pYcDE53-1 has *CDC53* expressed from the *ADH1* promoter. The 1.1-kb *Hin*dIII DNA fragment of pK34-1 (12) was filled in by Klenow fragment and ligated into the filled-in *Sma*I-*Pvu*II fragment of YEp24 to generate YEp34-1. Plasmid pCDC53-20 was constructed by deleting a 250-bp *Kpn*I DNA fragment from the coding region of *CDC53* within E3a. This plasmid now encodes a nonfunctional mutant Cdc53p lacking amino acid residues 582 to 665.

To construct pNM53, an *Sph*I-*Hin*dIII DNA fragment from plasmid pYcDE-53 containing the *ADH1* promoter and a portion of *CDC53* was ligated into the *Sph*I-*Hin*dIII sites of pGEM-7 (Promega Corp.) to create plasmid pNM53Δ. An *Apa*I-*Nco*I DNA fragment which contains the *ADH1* promoter and a portion of *CDC53* was excised from plasmid pNM53Δ and ligated into the *Apa*I-*Nco*I sites of pMT711 (kindly provided by M. Tyers), to create pNM53. Plasmid pNM53 contains the full-length *CDC53* gene under the control of the *ADH1* promoter.

Plasmid YEpGALHis34 was constructed as follows. An *Nde*I-*Bgl*II DNA fragment from plasmid pMT729 (kindly provided by M. Tyers) containing the *CDC34* coding region was ligated into the *Nde*I-*Bam*HI sites of pET15b (Novagene). This plasmid is called pHis34 and contains a fusion gene encoding a Cdc34p with six histidine residues at the N terminus of the protein. A *Kpn*I-*Bam*HI DNA fragment containing the *GAL10* promoter region from plasmid pSJ101 was ligated into the *Kpn*I-*Bam*HI sites of pGEM3 (Promega Corp.) to generate pGEM-GAL10. An *Nco*I-*Hin*dIII DNA fragment encoding the His-tagged Cdc34p from plasmid pHis34 was then ligated into the *Nco*I-*Hin*dIII sites of plasmid pGEM-GAL10 to generate pGEM-GALHis34. Plasmid pRSGAL-His34 was generated by ligating a *Kpn*I-*Hin*dIII DNA fragment containing the *GAL*-His-*CDC34* region of plasmid pGEM-GALHis34 into the *Kpn*I-*Hin*dIII sites of pRS316. Finally, YEpGALHis34 was constructed by replacing a *Sca*I DNA fragment containing centromere sequences from pRSGALHis34 with the *Sca*I DNA fragment containing the 2μm plasmid replication origin from YEp24.

DNA sequencing. The DNA sequence of the *CDC53* gene was determined as described previously (12). Two series of overlapping deletions extending into either side of *CDC53* were generated by exonuclease III digestion (15) using pGEM53-8 and pGEM53-9 as templates, and the DNA sequence analysis was performed with Sequenase (U.S. Biochemical) as instructed by the manufacturer.

Antibody generation and Western blot (immunoblot) analysis. Anti-Cdc4 and anti-Cdc53 antisera were prepared in New Zealand White rabbits, using Cdc4p and Cdc53p immunogens produced in *Escherichia coli* as described previously (11). First, plasmid pATH4-5 was created by ligating an 800-bp *Eco*RV DNA fragment from pCDC4-35 (52) into the *Sma*I site of pATH1 (25). Plasmid pATH53-2 was created by ligating the 1.0-kb *Xba*I-*Hin*dIII DNA fragment from pGEM53-8 into the *Xba*I-*Hin*dIII sites of pATH2 (25). When pATH4-5 and pATH53-2 were transformed into *E. coli* DH-1 and RR1 (38), respectively, the encoded regions of Cdc4p and Cdc53p were produced as fusions to bacterial TrpE as described previously (11). These fusion proteins were purified and injected into rabbits to produce antisera to Cdc4p and Cdc53p. Antibodies to Cdc4p and Cdc53p were affinity purified prior to Western analysis as described previously (11).

Yeast cell lysates were prepared and Western analysis was performed as described previously (11) except that during the electrophoretic transfer of proteins from the sodium dodecyl sulfate (SDS)-polyacrylamide gel onto polyvinylidene fluoride membranes, 10 mM Tris-HCl (pH 7.0) was replaced with 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11.0). Affinity-purified anti-Cdc4, anti-Cdc34 (11), and anti-Cdc53 were used at a final concentration of 1/1,000, and the antibodies were visualized by enhanced chemiluminescence (Amersham) as instructed by the manufacturer.

Purification of His-tagged Cdc34p by nickel chromatography. Yeast cell lysates were prepared as described above except that the cells were lysed in 50 mM NaCl–50 mM Tris-HCl (pH 7.5)–0.5 mM EDTA–1% Triton. Lysate containing 10 mg of protein was incubated for 5 min with 200 μl of Ni^{2+}-nitrilotriacetic acid agarose beads (Qiagen) that had been previously equilibrated with 50 mM NaCl–50 mM Tris-HCl (pH 7.5)–30 mM imidazole. The beads were then washed repeatedly with 2 ml of equilibration buffer containing 30 mM imidazole. The

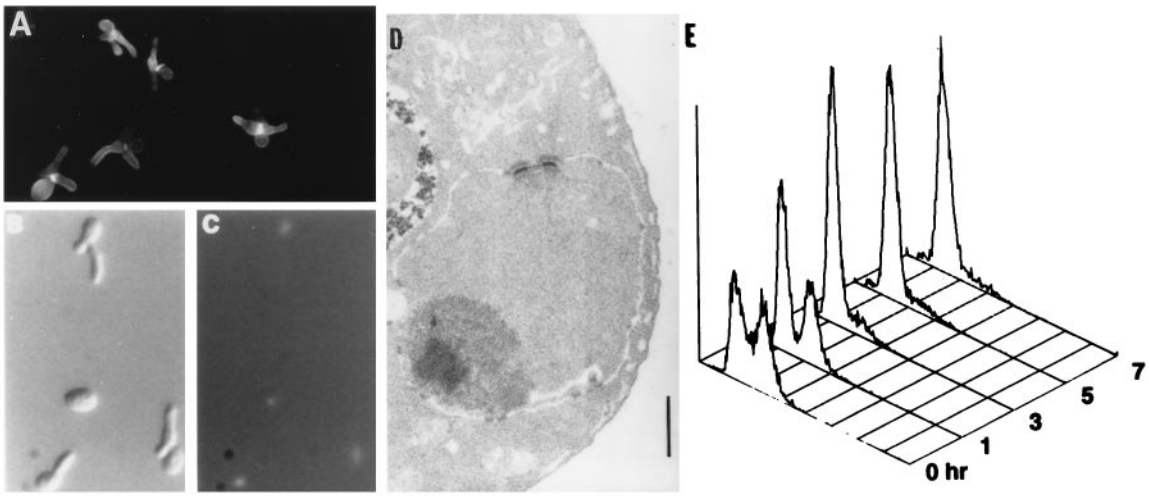

FIG. 1. Phenotypes of *cdc53* mutants. Cells were grown in YM-1 medium overnight at 23°C to a density of 10^6/ml and then shifted to 36°C for 3 h or as noted. (A) Terminal morphology of *cdc53-1* strain JPT175. Cells were shifted to 36°C for 6 h, stained with Calcofluor as described previously (34), and visualized by fluorescence microscopy using the UV filter set. (B and C) Cells of strain KJB1 were fixed in ethanol and stained with propidium iodide. Cells were then visualized by differential interference contrast microscopy (B) or fluorescence microscopy with appropriate filters (C). (D) Cells of *cdc53-2* strain WX70.3c have duplicated but unseparated SPBs in their nuclear envelopes at arrest, as shown by serial thin-section electron microscopy. The pair of SPBs shown lie opposite the darkly stained nucleolus. Scale bar, 0.5 μm. (E) Cells of *cdc53-2* strain KJB1 arrest with a G_1 content of DNA. Cells were removed at hourly intervals after a shift to 36°C, fixed in ethanol, stained with propidium iodide, and subjected to flow cytometry with a FACScan analyzer.

protein bound to the beads was then subjected to Western analysis as described previously (11).

Nucleotide sequence accession number. The sequence of *CDC53* has been assigned GenBank accession number U43564.

RESULTS

Isolation and characterization of *cdc53* mutants. The presence of multiple, elongated buds at 36°C is a characteristic of cells that contain a temperature-sensitive mutation in either *CDC4* or *CDC34* (5, 6, 14). Therefore, to identify other genes involved in the same process(es), we screened a collection of Ts^- mutants (1) microscopically for those that formed abnormal cell shapes under nonpermissive conditions (growth at 36°C). Three mutants, called JPT175, JPTA1528, and JPTA1529, formed multiple, elongated buds (Fig. 1A). Backcrosses revealed a single temperature-sensitive mutation in each strain, and these mutations fell into a single complementation group differing from all previously characterized *cdc* mutations. This complementation group was termed *CDC53*, and the alleles found in JPT175, JPTA1529, and JPTA1528 were designated *cdc53-1*, *cdc53-3*, and *cdc53-4*, respectively. Staining of nuclear DNA in the arrested cells indicated the presence of a single nucleus (data not shown, but see Fig. 1B and C). These features of the terminal phenotype were indistinguishable from those of mutants defective for *CDC4* or *CDC34* (5, 6, 14). However, the *cdc53-1* mutation was genetically mapped to a position about 24 centimorgans distal to *cdc2* on the left arm of chromosome IV (31), which differs from the map positions of *cdc4* and *cdc34*.

Independent of the above analysis, a second collection of Ts^- mutants was screened for defects in microtubule organization (49). The mutation found in strain ts328, when passed through backcrosses, failed to maintain the original defect in microtubule organization but caused cells to arrest with a single nucleus and multiple, elongated buds (Fig. 1B and C). Complementation analysis indicated that this mutation (designated *cdc53-2*) was allelic with *cdc53-1*. Further analysis of this mutant collection also revealed temperature-sensitive mutations with similar phenotypes that failed to complement *cdc34-2*. Two of these strains were found to contain previously unknown alleles of *CDC34* that were designated *cdc34-4* and *cdc34-5* (28).

More detailed characterization of cells arrested by *cdc53* mutation revealed additional similarities to the phenotypes of *cdc4* and *cdc34* mutants. Two key features of the arrest seen in *cdc4* and *cdc34* mutants are the failure to separate the SPBs (5, 6) and to initiate nuclear DNA replication (14). The behavior of the SPBs in the *cdc53* mutants was examined by electron microscopy. Examination of serial sections through 20 cells of a *cdc53-2* strain arrested for 3 h at 36°C revealed in every case that the SPB had undergone duplication but that the two SPBs remained in a side-by-side configuration, failing to separate from one another and form a spindle (Fig. 1D). Cells that contained the *cdc53-1* mutation, on the other hand, arrested as a mixed population containing cells with unseparated SPBs together with cells that had formed mitotic spindles when incubated under nonpermissive conditions. However, cells containing *cdc53-1* arrest with multiple, elongated buds indistinguishable from those of *cdc53-2* mutants. These results suggest that the *cdc53-1* allele causes a leaky cell cycle arrest rather than causing cells to arrest at a different stage of the cell cycle.

We performed flow cytometry to determine the nuclear DNA content of *cdc53* mutants under nonpermissive conditions. Within 3 h of shift to 36°C, the *cdc53-2* mutant arrested with a G_1 DNA content (Fig. 1E). The fact that cells from the initial S and G_2/M peaks were chased into the G_1 peak during incubation at 36°C indicates that cells deficient in *CDC53* function can complete nuclear DNA replication and mitosis but are unable to initiate a new round of DNA replication. Thus, in each respect analyzed, the *cdc53* defects are indistinguishable from those caused by *cdc4* and *cdc34*.

Synthetic lethal interactions between *cdc4*, *cdc34*, and *cdc53*. Functional interactions between gene products can sometimes be detected by synthetic lethality, in which a combination of alleles that are independently nonlethal cause lethality under the same conditions (17). To determine whether synthetic lethal interactions occur between *cdc4*, *cdc34*, and *cdc53*, strains containing these mutations were crossed to each other, and the

TABLE 2. Synthetic lethality of *cdc4*, *cdc34*, and *cdc53* mutant combinations

Cross	Mutation in cross		Spore recovery (live/total)			
	a	b	wild type	a	b	a,b
SJ1026-1B × MGG15	*cdc4-3*	*cdc34-2*	17/17	18/19	19/19	0/17
SJ1026-7B × SJ1080-8C	*cdc4-3*	*cdc53-1*	25/25	25/27	24/27	0/25
SJ1012-4 × SJ1080-6C	*cdc4-5*	*cdc53-1*	27/27	22/27	27/27	0/27
MGG11 × MGG10	*cdc34-2*	*cdc53-1*	13/13	23/25	22/25	0/12
H3C1B5 × SJ1078-2B	*cdc3-1*	*cdc4-3*	16/17	7/10	10/11	11/16
H3C1B5 × SJ1098-3D	*cdc3-1*	*cdc34-2*	9/10	15/16	16/16	10/10
H122-12-3 × SJ1078-2B	*cdc10-1*	*cdc4-3*	9/9	18/19	19/19	9/9
H122-12-3 × SJ1078-3D	*cdc10-1*	*cdc34-2*	8/8	18/18	18/18	8/8
6353-14-3 × 7414-2-3	*cdc4-1*	*cdc28-1*	27/29	27/29	26/29	27/29
6353-4-1 × 5579-5-2	*cdc4-1*	*cdc36-16*	9/9	21/21	21/21	9/9
MGG12 × H2C2A-1	*cdc53-1*	*cdc2-2*	15/15	125/125	125/125	15/15
SJ1078-2B × 5566-1-1	*cdc4-3*	*cdc39-1*	14/16	4/5	4/5	16/16

resulting diploids were sporulated to test for viability of the spores at 23°C, a permissive temperature for these mutations (Table 2). Although the viability of spores containing any one of these temperature-sensitive alleles alone was over 90%, no spore containing any pair of mutant alleles among these three genes was ever recovered. Furthermore, when the inviable spores predicted to contain two mutations were examined microscopically, most of the dead spores had germinated and developed multiple, elongated buds typical of strains mutant for any one of these genes after transfer to nonpermissive temperature. In contrast, double-mutant combinations were viable from crosses between mutants for any one of these three genes (*cdc4*, *cdc34*, and *cdc53*) and a number of other temperature-sensitive cell cycle mutants (Table 2). Liang et al. (27) have also shown that synthetic lethality is not observed in crosses between *cdc34* and other genes required for the initiation of DNA replication, including *cdc6*, *orc2*, and *orc5*.

Molecular characterization of *CDC53*. The wild-type *CDC53* gene was cloned by complementation of the temperature sensitivity caused by *cdc53-3*. After transformation with a YEp24-based yeast genomic library (7), one transformant that grew at 36°C was recovered. Plasmid E3a, containing an 8.2-kb insert, was isolated from this transformant; complementation analysis using various subcloned fragments demonstrated that the *cdc53*-complementing ability of E3a could be localized to a 3.6-kb *Eco*RI fragment (Fig. 2).

To demonstrate that the complementing plasmids contained *CDC53* rather than a high-copy-number suppressor, the diploid MGG47 (heterozygous for *cdc53-1*) was transformed with DNA from plasmid pCDC53-9 (see Materials and Methods) after digestion with *Apa*I to target integration to the *CDC53* locus. Two transformants, MGG47-1 and MGG47-2, which should contain a tandem duplication of *CDC53* and a copy of *TRP1* at the *CDC53* locus, were sporulated and subjected to tetrad analysis. The integrated plasmid sequences in these two strains segregated away from the *cdc53-1* allele in 11 of 11 and 9 of 9 tetrads, respectively, demonstrating that the plasmid sequences had indeed integrated at a genomic location tightly linked to *cdc53-1*. Southern analysis of yeast genomic DNA with a probe consisting of a 2.3-kb *Cla*I-*Bst*EII DNA fragment internal to the *CDC53* coding region (see below) did not indicate the presence of any cross-hybridizing DNA within the yeast genome (29). These results indicate that we have cloned the wild-type allele of *CDC53* and that there are no other genes closely related to *CDC53* within the yeast genome.

We next determined whether the phenotype of cells containing a completely nonfunctional *CDC53* gene was similar to the arrest phenotype of the *cdc53* temperature-sensitive mutants. Null alleles of *CDC53* were created by transforming diploid strains MGG3 and MGG48 with *Eco*RI-digested pGEM53-ΔBglII::*HIS3* (see Materials and Methods). This results in the replacement of 1.6-kb of the *CDC53* coding region (see below) by *HIS3*. After verifying by Southern analysis that one transformant from each strain contained the expected replacement, these strains were sporulated and subjected to tetrad analysis. In a total of 27 tetrads, only two viable spores (none of which was His^+) were recovered from each tetrad, indicating that *CDC53* is an essential gene. Microscopic examination revealed that most of the inviable spores had progressed through several rounds of cell division to form a microcolony before arresting. Each cell in these microcolonies had developed multiple, elongated buds that were indistinguishable from the aberrant buds seen after the cell cycle arrest of *cdc53* temperature-sensitive mutants. Thus, the phenotype of the *cdc53* temperature-sensitive mutations suggests that these alleles suffer a loss-of-function defect upon transfer to the restrictive temperature.

The DNA sequence was determined for the complementing 3.6-kb *Eco*RI fragment, revealing an open reading frame (ORF) for a protein of 815 amino acids with a calculated molecular mass of 94 kDa (Fig. 2). The most striking feature of the predicted protein is its basic nature (predicted pI = 8.7). Comparison of the protein sequence with those in GenBank with BLAST (2) identified two proteins encoded by *Caenorhabditis elegans* genes that are related to Cdc53p, the *cul-1* product (23; accession number Z35639) and the *cul-4* product (23; accession number U29536). Other sequences similar to the derived Cdc53p sequence are an ORF identified on chromosome I of *Schizosaccharomyces pombe* (accession number Z54142) and a mammalian protein thought to be involved in vasopressin-dependent calcium mobilization, VACM-1 (4; accession number S78157). Figure 3 shows an alignment of the products of *CDC53* and *cul-1* with the Cdc53p-like protein of *S. pombe* and the VACM-1 protein. Although Cdc53p shows only about 25% identity to each of these proteins, all four proteins are identical at over 50 positions, which are clustered. A similarity search of the EST sequence library also indicates that at least four additional distinct mammalian proteins are members of this family (23). Thus, the Cdc53/CUL-1 protein family is widely conserved.

A TBLAST search of the predicted sequence of Cdc53p against the Saccharomyces Genome Database (Stanford University) also revealed that there are two other ORFs in the yeast genome with similarity to Cdc53p, Ygr003p and Yj1047p (Fig. 4). Again the sequences are not closely related overall,

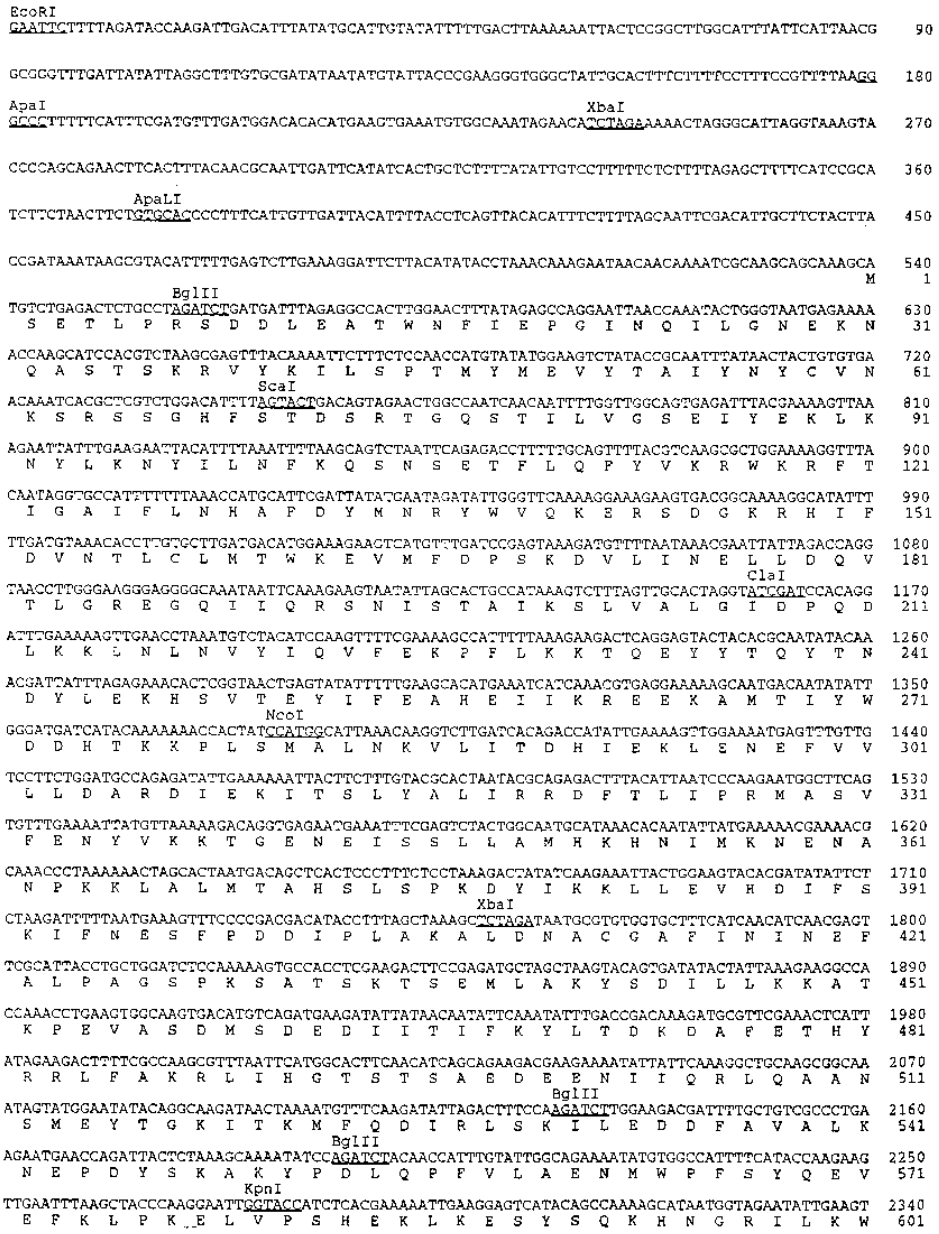

FIG. 2. DNA sequence of *CDC53* and the predicted amino acid sequence of its gene product.

the regions of similarity are clustered and are predominantly located within the COOH termini of the gene products. Disruption of either of these ORFs does not lead to inviability, indicating that Cdc53p is the only essential member of this family in *S. cerevisiae* (29).

Suppression of mutations in *CDC4* and *CDC34* by overexpression of *CDC53*. Because our analysis for synthetic lethality had suggested that *CDC4*, *CDC34*, and *CDC53* might cooperate in conferring a common function, we used the cloned genes to seek additional evidence for relevant interactions. The three wild-type genes, including their putative promoter regions, were placed under the control of a yeast *GAL* promoter in high-copy-number (2μm-based) plasmids (see Materials and Methods). These plasmids were then individually transformed into strains carrying a *cdc4-3*, *cdc4-5*, *cdc34-2*, or *cdc53-1* mutant allele. When the transformants were transferred to 34°C, overexpression of *CDC53* was seen to suppress the lethality caused by either *cdc4-5* (with or without galactose induction) or by *cdc34-2* (with galactose induction) (Table 3). In contrast, overexpression of *CDC34* on galactose suppressed *cdc53-1* but failed to suppress either *cdc4* allele. In fact, overexpression of *CDC34* caused *cdc4* mutants to become inviable under normally permissive conditions (23°C). Modest overexpression of *CDC4* suppressed the *cdc53-1* mutation at 34°C, but higher-level (galactose-induced) expression of *CDC4* caused cells mutant for either *cdc34-2* or *cdc53-1* to become inviable at 23°C (Table 3). Interestingly, while overexpression of *CDC53* could suppress the temperature sensitivity of the *cdc4-5* strain, it did not suppress the *cdc4-3* allele (Table 3) or several other *cdc4* alleles (*cdc4-1*, *-2*, *-4*, *-6*, and *-7*) that were tested (22). The positions of these latter mutations in *CDC4* have been mapped to the region encod-

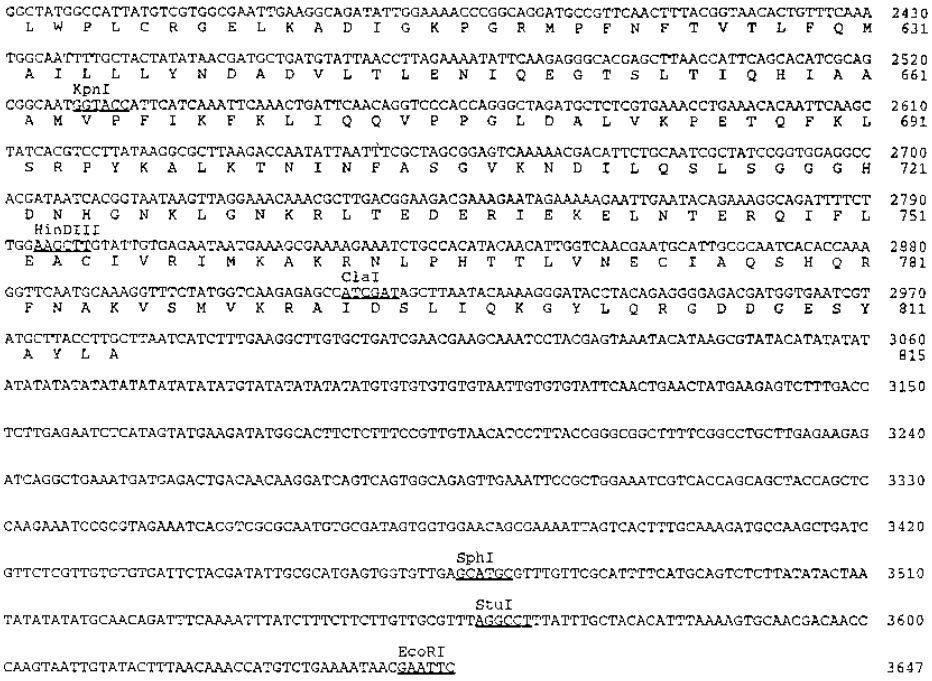

FIG. 2—*Continued.*

ing the WD-40 repeats of Cdc4p, whereas the *cdc4-5* allele maps to a nonrepetitive amino-terminal portion of Cdc4p that is essential for function (22). These results suggest that Cdc53p interacts with this amino-terminal portion of Cdc4p rather than with the WD-40 repeats. Taken together, the suppression data provide additional strong genetic evidence that the encoded proteins interact.

Characterization of Cdc53p. To allow assays of Cdc53p, rabbit antibodies were generated against a TrpE-Cdc53p fusion (see Materials and Methods). In yeast extracts, these antibodies recognized protein species of 92 and 98 kDa (Fig. 5, lane 1), similar to the predicted mass (94 kDa) of Cdc53p. Several results indicate that these antibodies recognize Cdc53p and that both the 92- and 98-kDa proteins are products of *CDC53*. First, an extract from cells overexpressing wild-type *CDC53* has elevated levels of both the 92- and 98-kDa proteins (Fig. 5, lanes 4 and 5), whereas extracts from cells containing *cdc53-1* have decreased levels of the 92-kDa protein (Fig. 5, lane 6). Second, an extract from cells expressing a nonfunctional form of Cdc53p that contains an internal deletion of amino acid residues 582 to 665 (expressed from pCDC53-20) contains an 80-kDa protein that is recognized by the antibodies (Fig. 5, lane 2). Expression of *CDC53**, encoding a protein in which the carboxyl-terminal 23 amino acids are replaced by vector-encoded sequence (expressed from either pFUS53-3 or pYcDE-53; Fig. 2 and Materials and Methods) contained an increased level of a 98-kDa fusion protein that is consistently greater than the level of Cdc53p achieved by overexpressing wild-type *CDC53* (Fig. 5, lane 3). Interestingly, the 98-kDa Cdc53p has recently been shown to be ubiquitinated (48). However, overproduction of the *CDC53**-encoded fusion protein does not lead to the appearance of a higher-molecular-mass form equivalent to the 98-kDa protein seen with overexpression of wild-type *CDC53*, suggesting that the increased levels of this protein might be due to removal of signals required for its ubiquitination and targeted degradation.

Copurification of Cdc4, Cdc34, and Cdc53. We next sought direct evidence that the three proteins Cdc4p, Cdc34p, and Cdc53p interact. We constructed plasmid YEpGALHis34, which encodes a 6-His-tagged Cdc34p (see Materials and Methods) that can rescue cells containing a *cdc34* null mutation. YL10-1 cells containing YEpGALHis34 were grown at 36°C on either galactose- or glucose-containing medium; lysates were prepared as described in Materials and Methods, and the His-tagged Cdc34p was purified by nickel affinity chromatography. Lysate and Ni^{2+}-nitrilotriacetic acid agarose (with protein bound to the nickel beads) were subjected to SDS-polyacrylamide gel electrophoresis, and Western blot analysis was performed with anti-Cdc4, anti-Cdc34, and anti-Cdc53 antibodies. Samples were treated such that quantitative binding of a protein to the nickel beads should result in a 20-fold enrichment of the protein relative to the abundance of the protein in the initial lysates. As can be seen in Fig. 6, His-tagged Cdc34p is highly enriched after nickel affinity chromatography, and no cross-reacting signal can be detected in lysates lacking the His-tagged Cdc34p. Comparison of exposure intensities indicates that about 80% of the His-tagged Cdc34p is recovered from the nickel beads. While Cdc4p is undetectable in lysates, it copurifies with His-tagged Cdc34p and can be easily detected in the protein bound to the nickel beads (Fig. 6). About 25% of the 92-kDa species of Cdc53p is also found bound to the nickel beads, but only in the presence of the His-tagged Cdc34p (Fig. 6). Thus, even in the presence of native Cdc34p, substantial fractions of cellular Cdc4p and Cdc53p are bound to the nickel beads by binding to the His-tagged Cdc34p (Fig. 6). Although the 98-kDa species of Cdc53p can be detected in the absence of His-tagged Cdc34p, it is also highly enriched in the presence of the His-tagged Cdc34p protein. These interactions are specific, as a number of cross-reacting bands seen with the anti-Cdc4 and anti-Cdc34 antibodies are eliminated by this purification procedure. Together, these data suggest that Cdc4p, Cdc34p, and Cdc53p are associated in a multiprotein complex.

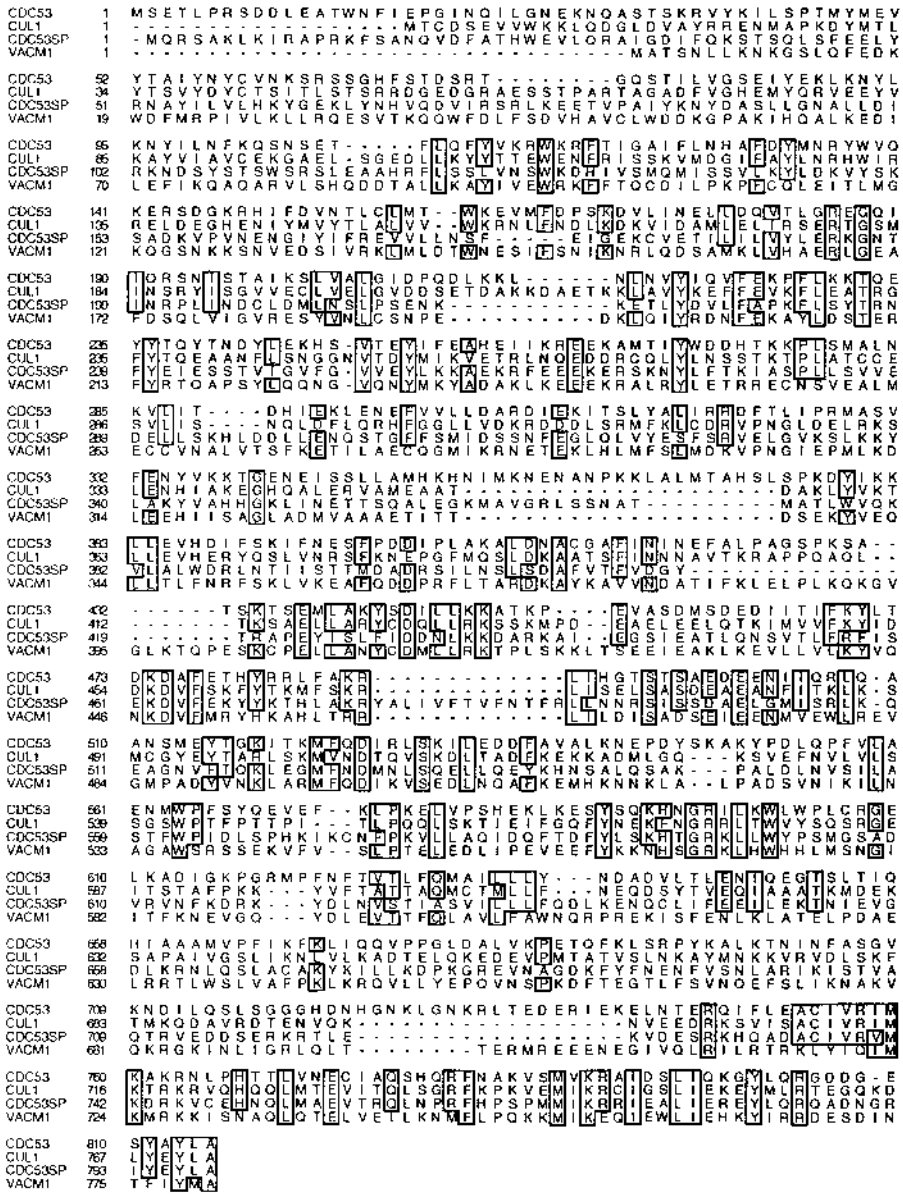

FIG. 3. Comparison of the Cdc53p-related proteins from *C. elegans*, *S. pombe*, and rabbits. Protein sequences were aligned by using the multiple sequence alignment program MACAW (40) and the SeqVu software program (Garvan Institute of Medical Research). Positions where Cdc53p is identical to at least two of the other three proteins are boxed.

DISCUSSION

In *S. cerevisiae*, *CDC4* and *CDC34* are required for the transition from late G_1 to S phase (for a review, see reference 33). Cells mutant for *CDC4* or *CDC34* can undergo the Start-dependent events of SPB duplication, bud emergence, and activation of MBF- and SBF-dependent transcription (5, 6, 14, 24). However, these mutants fail nuclear DNA replication, spindle formation, and cytokinesis. We have identified several alleles of the novel essential gene *CDC53* that cause identical phenotypic traits, suggesting that *CDC53* is required for the same cell cycle function as *CDC4* and *CDC34*. Further evidence for a functional relationship among these three genes is provided by striking genetic interactions among the mutant alleles. First, *cdc53-1* is synthetically lethal with *cdc4-3*, *cdc4-5*, and *cdc34-2* at 23°C. This synthetic lethal effect apparently results from a loss of function that is very similar to that caused by temperature sensitivity for any one of the three genes, because cells suffering this synthetic lethality arrest development with the same terminal morphology as that of the individual mutants. Second, overexpression of *CDC53* suppresses the *cdc34-2* and *cdc4-5* temperature-sensitive alleles, and overexpression of *CDC34* suppresses *cdc53-1*. Depending on the level of overexpression achieved, *CDC4* can either suppress *cdc53-1* (multiple copies expressed from the *CDC4* promoter) or enhance the *cdc53-1* phenotype (multiple copies expressed from the *GAL10* promoter).

Together, these results suggest that the Cdc53p protein interacts with Cdc4p and Cdc34p in a common function that mediates the transition from late G_1 into S phase. Decreases in this function resulting from combining mild mutations for any

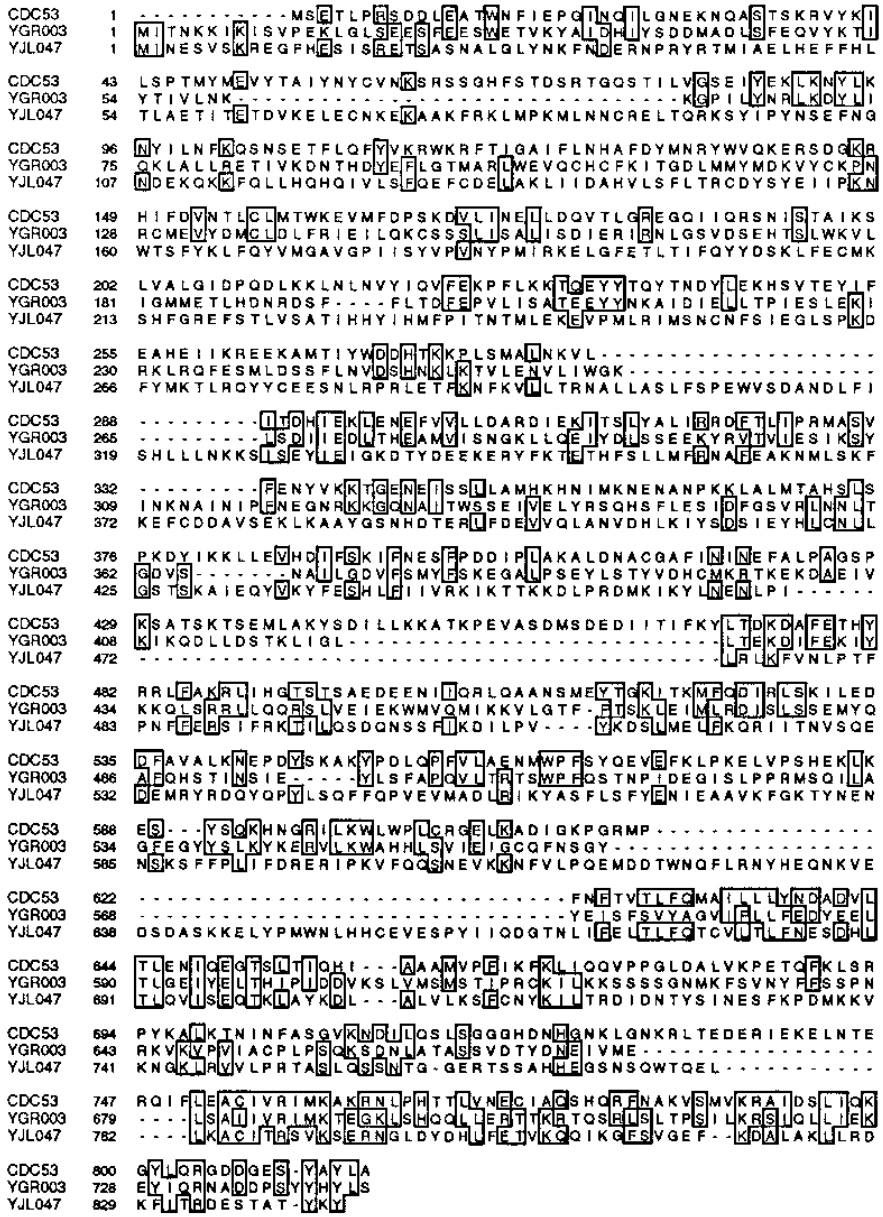

FIG. 4. Comparison of Cdc53p with Ygr003p and Yjl047p. Protein sequences were aligned as described in the legend to Fig. 3. Positions where two of the three proteins are identical are boxed.

TABLE 3. Effects of overexpressing *CDC4*, *CDC34*, and *CDC53* in *cdc4*, *cdc34*, and *cdc53* mutants[a]

Mutation[b]	Vector[c]		*CDC4*		*CDC34*		*CDC53*	
	Raffinose	Galactose	Raffinose	Galactose	Raffinose	Galactose	Raffinose	Galactose
None	+	+	+	+	+	+	+	+
cdc4-3	−	−	+	+	−	−[d]	−	−
cdc4-5	−	−	+	+	−	−[d]	+	+
cdc34-2	−	−	−	−[d]	+	+	−	+
cdc53-1	−	−	+	−[d]	−	+	+	+

[a] Growth monitored at 34°C unless otherwise indicated.
[b] Strains: wild-type, Ts$^+$ revertant of SJ1026-7B; *cdc4-3*, SJ1026-7B; *cdc4-5*, SJ1012-4; *cdc34-2*, SJ1098-3D; *cdc53-1*, SJ1080-4D.
[c] Plasmids: vector, pSJ101; *CDC4*, pSJ4101; *CDC34*, pFUS34; *CDC53*, pFUS53-3.
[d] Growth monitored at 23°C.

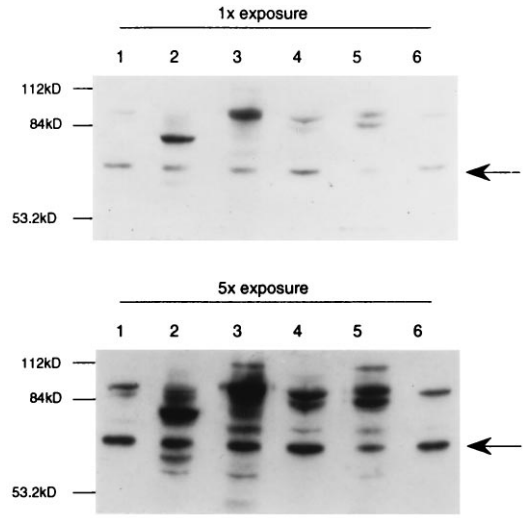

FIG. 5. Western blot analysis of Cdc53p. Cells were grown at 23°C. Protein was extracted and subjected to Western analysis with anti-Cdc53 antibodies. Lanes: 1, Y382; 2, Y382(pCDC53-20); 3, Y382(pYcDE-53); 4, Y382(pCDC53-11); 5, Y382(pNM53); 6, MGG12 (*cdc53-1*). Arrows indicate positions of a cross-reacting protein that served as the loading control. Approximately equal amounts of protein were loaded into each lane as also determined by Coomassie blue staining of identical samples.

two members of the set to generate synthetic lethality or from a stronger mutation affecting any one member yield the same terminal phenotype. The interaction between *CDC53* and *CDC4* is particularly intriguing. The *cdc4-5* allele that can be suppressed by overexpressing *CDC53* is located within a region of *CDC4* encoding an essential domain of Cdc4; in contrast, *CDC53* does not suppress a number of alleles that have been mapped to the portion of *CDC4* encoding WD-40 repeats. These data suggest that Cdc53p binding to Cdc4p is weakened in the protein encoded by *cdc4-5* and that overproduction of Cdc53p enhances its interaction with this essential domain of Cdc4p. Finally, direct evidence that the three proteins reside in a single complex comes from the copurification of Cdc4p and Cdc53p with His-tagged Cdc34p by nickel chromatography. Together, these data demonstrate that these three proteins interact strongly with one another.

The present evidence clearly indicates that Cdc53p functions in conjunction with Cdc34p, which is a ubiquitin-conjugating

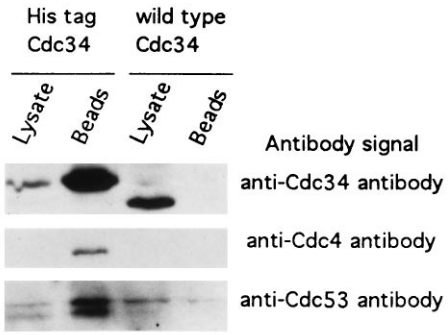

FIG. 6. Copurification of Cdc4p, Cdc34p, and Cdc53p. Nickel chromatography was used to purify His-tagged Cdc34p from strain YL10-1 harboring YEpGALHis34. Mock purification was performed with strain Y382 lacking His-tagged Cdc34p. Western analysis was performed on material bound to the beads as well as the original lysate, using an anti-Cdc4, anti-Cdc34, or anti-Cdc53 antibody.

enzyme (12). Ubiquitin is a small, highly conserved protein that is found as a posttranslational modification of substrate proteins (for a review, see reference 19). Ubiquitin is activated in an ATP-dependent manner by a ubiquitin-activating or E1 enzyme, which forms a thioester linkage between the COOH-terminal glycine of ubiquitin and a cysteine within the E1 enzyme. The E1 enzyme then transfers the ubiquitin to any one of a family of proteins known as ubiquitin-conjugating enzymes, again via a thiolester linkage. Finally, often in a manner dependent on another activity known as an E3 enzyme or ubiquitin ligase, ubiquitin is attached to a substrate protein via an isopeptide linkage, thereby targeting the substrate for proteolysis by the proteasome.

Although the exact function of Cdc53p remains to be established, it is likely that this protein is involved in controlling the ubiquitin-dependent degradation of cell cycle mediators in concert with Cdc34p. The known substrates of Cdc34p include both the G_1 cyclins (8, 47, 48) and the cyclin-dependent kinase inhibitors Far1p and Sic1p (30, 41). One possibility is that Cdc53p controls the level of activity of the Cdc34p. Alternatively, Cdc53p may be necessary to bring Cdc34p in proximity to its substrates. A third possibility is that Cdc53p functions as an E3 protein. Recently, Scheffner et al. (39) demonstrated that members of at least one family of E3 proteins directly form a thiolester linkage with ubiquitin and presumably go on to form an isopeptide linkage between ubiquitin and the substrate protein. While a mechanism of this type may be unlikely in the case of Cdc53p-like proteins (members of this family do not contain the conserved cysteine [Fig. 3] that serves as the ubiquitin acceptor in the recently described family of E3 proteins [18]), it cannot be ruled out at this time.

Sequence comparison shows that Cdc53p is a member of a family of proteins present in a diversity of organisms, including *S. pombe*, *C. elegans*, and humans. Not only are these related proteins present in these various organisms, but multiple homologs are known to exist in both *C. elegans* and humans. Although the functions of these proteins are unknown, their amino acid sequence similarity to Cdc53p suggests that they might also serve to regulate protein degradation events, possibly by controlling the activity of other ubiquitin-conjugating enzymes. Although the cellular multiplicity of Cdc53p-like proteins may indicate a diversity of functions for such proteins, at least some members of this family share with Cdc53p a critical role in cell cycle control events in other organisms as well. The *cul-1* gene of *C. elegans* encodes a Cdc53p-like protein (23). Mutations in *cul-1* cause a defect in the ability of cells to arrest cell division during development. Thus, CUL-1 acts as an inhibitor of entry into the cell division cycle. Although this inhibitory role appears to be dissimilar to the stimulatory function of Cdc53p, we speculate that Cdc53p-like proteins may share a common role in controlling the abundance of cell cycle regulatory molecules that may either promote or oppose cell cycle progression in different organisms. Thus, Cdc53p and CUL-1 define a novel set of proteins and suggest a potential role for this large conserved protein family in controlling cell division events as regulators of ubiquitin-dependent protein degradation.

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